Virology 508 (2017) 170–179

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Residues F103 and M106 within the influenza A virus NS1 CPSF4-binding region regulate interferon-stimulated gene translation initiation

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ARTICLE INFO

Keywords:
Influenza A virus
Non-structural protein 1
Type I interferons
Interferon-stimulated genes
Polysomes

ABSTRACT

Influenza A virus (IAV) non-structural protein 1 (NS1) suppresses host innate immune responses by inhibiting type I interferon (IFN) production. We provide evidence that residues F103 and M106 in the CPSF4-binding domain of A/HK/1/68 [H3N2] NS1 contribute to post-transcriptional inhibition of antiviral IFN-stimulated genes (ISGs), thereby suppressing an antiviral type I IFN response. Recombinant (r) IAVs encoding F103L and M106I mutations in NS1 replicate to significantly lower viral titers in human A549 lung epithelial cells and primary type II alveolar cells. In A549 cells, rIAVs encoding these mutant NS1s induce higher levels of IFN-β production and are more sensitive to the antiviral effects of IFN-β treatment. qPCR characterization of polysomal mRNA, in the presence or absence of IFN-β treatment, identified a greater proportion of heavy polysome-associated ISGs including *EIF2AK2*, *OAS1*, and *MxA* in A549 cells infected with rIAVs encoding these CPSF4-binding mutant NS1s, in contrast to rIAV encoding wildtype NS1.

1. Introduction

Type I interferons (IFNs) are produced as part of the innate immune response to virus infections. Upon recognition of viral pathogen-associated molecular patterns (PAMPs), such as viral genomic material, pattern recognition receptors (PRRs) activate NF-κB, IFN regulatory factor (IRF) 3 and IRF7 to initiate the transcription of IFNs-α/β (Juang et al., 1998; Sato et al., 1998; Panne et al., 2007). IFN-α/β signaling through the type I IFN receptor, IFNAR, induces the expression of antiviral IFN-stimulated genes (ISGs), which encode proteins that target various stages of virus replication and establish an antiviral state in uninfected cells (Basler and Garcia-Sastre, 2002; Wang and Fish, 2012). As a result, many pathogenic viruses have evolved strategies to suppress a type I IFN response, to ensure successful viral replication (Basler and Garcia-Sastre, 2002; Wang and Fish, 2012). Indeed, in the absence of type I IFN signaling, e.g. in *IFNAR*$^{-/-}$ mice, viruses exhibit enhanced replication and cell to cell transmission, and invoke pathologies in the host (Garcia-Sastre et al., 1998a; Koerner et al., 2007; Arimori et al., 2013).

Influenza A viruses (IAVs) have caused several pandemics in humans (Fineberg, 2014) and zoonotic transmission of new strains, coupled with the ability of IAVs to undergo genetic reassortment, pose a serious threat to global health (Shinde et al., 2009; Garten et al., 2009). Moreover, genetic drift has led to the emergence of antiviral resistant IAVs (Hurt et al., 2009).

IAVs encode a multifunctional non-structural protein, NS1, which binds dsRNA and host proteins to enhance viral mRNA translation, while suppressing host mRNA processing and innate immune responses (Hale et al., 2008). Among its many host targets, NS1 interacts with the p85β subunit of phosphoinositide 3-kinase (PI3K) to modulate protein kinase B (AKT) phosphorylation (Hale et al., 2010), and inhibits the activity of the innate IFN-inducible antiviral effectors, protein kinase RNA-activated (PKR) and 2'-5'-oligoadenylate synthetase (2'5'-OAS) (Min et al., 2007; Min and Krug, 2006). Importantly, NS1 inhibits the induction and expression of type I IFNs, specifically IFNs-α/β. IAVs that do not encode NS1 are highly attenuated in models of infection where the type I IFN response is intact (Garcia-Sastre et al., 1998b; Kochs et al., 2009). NS1-binding to 5' triphosphate viral RNAs and the PRR, retinoic acid-inducible gene 1 (RIG-I), suppresses NF-κB and IRF3 activation to inhibit IFN-β mRNA transcription (Mibayashi et al., 2007; Ruckle et al., 2012). Furthermore, NS1-binding to cleavage and polyadenylation specific factor 4, 30 kD subunit (CPSF4) inhibits host IFN-β pre-mRNA maturation to block IFN production (Nemeroff et al., 1998; Kochs

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http://dx.doi.org/10.1016/j.virol.2017.05.009
Received 30 March 2017; Received in revised form 10 May 2017; Accepted 11 May 2017

et al., 2007; Das et al., 2008).

CPSF4 is a key component of the mRNA processing machinery that adds poly(A) tails to the 3' end of pre-mRNAs. 3' polyadenylation enhances mRNA stability and enables mRNA nuclear export and translation (Huang and Carmichael, 1996; Huarte et al., 1992; Peltz et al., 1987). NS1-binding to CPSF4 causes an accumulation of IFN-β pre-mRNAs in the nucleus of IAV-infected cells, and a G184R mutation, which abrogates NS1-CPSF4 binding restores mature IFN-β mRNA expression (Das et al., 2008). Two highly conserved residues, F103 and M106, have been identified in the NS1 of human IAVs that stabilize NS1-CPSF4 binding (Nemeroff et al., 1998; Kochs et al., 2007; Das et al., 2008). *In vitro*, F103S/L and M106I mutations weaken and completely abrogate NS1-CPSF4 binding, respectively. In transient transfection studies with NS1 expression plasmids, NS1s expressing F103 and M106, but not S/L103 and I106, were able to inhibit ISG expression (von Recum-Knepper et al., 2015) and the activation of the promoter regions of ISGs such as MxA and IFN-β following IFN-α treatment (Kochs et al., 2007). Microarray analysis of host gene expression in cells infected with rIAV encoding NS1-F103+M106 resulted in both up- and downregulation of host genes, whereas cells infected with rIAVs encoding NS1-M106I or NS1-F103L+M106I were less effective at regulating host gene expression (Billharz et al., 2009).

To examine the effects of NS1-CPSF4 binding on NS1-mediated antagonism of an antiviral IFN response we generated A/HK/1/68 [H3N2] rIAVs (rHK) expressing either intact NS1 or NS1 CPSF4-binding mutants with F103L and/or M106I mutations. We provide further evidence that both F103L and M106I mutations attenuate rIAV replication in A549 cells, and that rIAVs expressing these mutant NS1s are more sensitive to the antiviral effects of IFN-β treatment. Importantly, we show that these mutations in NS1 affect the expression of select polysome-associated antiviral ISGs, thereby supporting an IFN antiviral response.

2. Materials and methods

2.1. Cells and reagents

Human lung adenocarcinoma epithelial A549 cells, human embryonic kidney HEK293T cells, and Madin-Darby canine kidney MDCK cells were purchased from ATCC (VA, USA) and maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, MA, USA). Human primary type II lung alveolar cells were provided by Dr. John M. Nicholls and Dr. Michael C.W. Chan (University of Hong Kong, Hong Kong), and maintained at 37 °C and 5% CO_2 in small airway epithelial cell growth medium (SAGM, Lonza, Switzerland) supplemented with the SAGM SingleQUOTs kit (Lonza) containing bovine pituitary extract, epinephrine, transferrin, hydrocortisone, rhEGF, rh insulin, retinoic acid, T3, gentamicin/amphotericin-B, and fatty acid free bovine serum albumin (BSA). Human IFN-β-1a (Avonex, specific activity 1.2×10^7 U/mL) was provided by Darren P. Baker (BiogenIdec, MA, USA).

2.2. Generation of rIAVs by reverse genetics

Plasmids (pLLB) (Liu et al., 2009) encoding the eight A/Hong Kong/1/1968 [H3N2] (HK) gene segments (HA, NA, NP, NS, PA, PB1, PB2, M), and mutant NS genes: NS-F103L/M106I/F103L+M106I were provided by Dr. Earl G. Brown (University of Ottawa, Ontario, Canada). rIAVs were generated as previously described (Martinez-Sobrido and Garcia-Sastre, 2010). 1 μg of each pLLB plasmid encoding one of the eight HK gene segments was transfected into 5 x 10^5 HEK293T cells to generate rA/HK/1/68 [H3N2] (rHK) virus. pLLB-HK-NS-F103L, pLLB-HK-NS-M106I, and pLLB-HK-NS-F103L +M106I were used in place of pLLB-HK-NS to generate rHK-NS-F103L, rHK-NS-M106I, and rHK-NS-F103L+M106I respectively. Viral titers were determined by plaque assay. rIAV studies were approved by the biosafety committee of the University Health Network and were performed in biosafety level 2 (BSL-2) conditions.

2.3. IAV infection

2×10^5 A549 cells or primary human type II lung alveolar cells were seeded in 24-well plates overnight. A549 cells and primary human type II lung alveolar cells were washed twice with PBS and infected in triplicate with each of the rHK viruses at a multiplicity of infection (MOI) of 0.01 and 0.1, respectively. Viruses were diluted in 500 μL of the respective cell culture media in the absence of serum and in the presence of 0.5 μg/mL (primary alveolar cells) or 1 μg/mL (A549 cells) TPCK-trypsin. Medium was collected at the indicated times post-infection and viral titers were determined by plaque assay.

2.4. Plaque assay

5×10^5 MDCK cells were seeded in 6-well plates for 24 h. Media containing rIAVs were diluted in serum-free DMEM containing 1 μg/mL TPCK-trypsin. MDCK cells were washed twice with 2 mL of PBS and infected with 800 μL of serially diluted rIAV. Infected MDCK cells were incubated at 37 °C for 1 h to allow virus adsorption before the addition of 2 mL of 0.65% agarose diluted in serum-free DMEM in the presence of 1 μg/mL TPCK-trypsin. MDCK cells were then incubated at 37 °C for 72 h, and fixed using a 3:1 methanol: acetic acid solution. Plaques were enumerated to determine the viral titer, recorded as the number of PFU/mL of medium.

2.5. IFN-β ELISA

IFN-β production by rIAV-infected A549 cells was quantified using the Verikine human IFN-β enzyme-linked immunosorbent assay (ELISA) kit (PBL Assay Science, NJ, USA) following the manufacturer's protocol. Media containing viral progeny were stored at −80 °C prior to use.

2.6. RNA extraction and cDNA synthesis

Cellular RNA was extracted and purified from infected A549 cells using the RNeasy Mini Kit (Qiagen, Netherlands) following the manufacturer's protocol. cDNA was synthesized using 1 μg/sample of RNA, random primers, and M-MLV reverse transcriptase (Invitrogen), following the manufacturer's protocol.

2.7. Characterization of polysomal RNA

Linear 5–55% (w/v) sucrose gradients were made by diluting a 55% (w/v) sucrose solution (filter sterilized) in gradient buffer and sequentially layering 6 mL fractions of 55%, 45%, 35%, 25%, 15%, and 5% sucrose in 25 × 89 mm polyallomer centrifuge tubes (Beckman Coulter, CA, USA) on dry ice. Gradient buffer contained 20 mM HEPES (pH 7.6), 0.1 M KCl, 5 mM $MgCl_2$, 10 μg/mL CHX (Sigma-Aldrich), 0.1x EDTA-free protease inhibitor cocktail (Roche, Germany), and 10 units/mL RNaseOUT recombinant ribonuclease inhibitor (Invitrogen). Sucrose gradients were stored at −80 °C and thawed at 4 °C overnight before use. 1.5 x 10^7 A549 cells were left uninfected or infected with each rHK virus at a MOI of 0.01. At 12 h post-infection, uninfected and infected A549 cells were left untreated or treated with 1×10^3 U/mL of human IFN-β-1a for 24 h before sucrose gradient centrifugation. Uninfected or rHK-infected A549 cells were treated with 100 μg/mL CHX for 5 min at 37 °C and then washed twice with ice-cold PBS containing 100 μg/mL CHX. Cells were lysed on ice using 500 μL of lysis buffer containing 5 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 1.5 mM KCl, 1× EDTA-free protease inhibitor cocktail, 100 μg/mL CHX, 2 mM DTT, 100 units of RNaseOUT, and 0.5% Triton X-100. Cell